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Genetic Analysis of Cold Hardiness in a Population of Norton (Vitis Aestivalis) and Cabernet Sauvignon (Vitis Vinifera) Hybrids

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GENETIC ANALYSIS OF COLD HARDINESS IN A POPULATION OF NORTON (*VITIS AESTIVALIS*) AND CABERNET SAUVIGNON (*VITIS VINIFERA*) HYBRIDS

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By

Daniel Adams

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ABSTRACT

Vitis aestivalis derived Norton is an American grape species common throughout the Midwest. Norton grapes are known for their disease resistance, high antioxidant content and cold hardiness; however, they typically have a lower wine quality than European varieties like Cabernet Sauvignon. Thus, there is a desire for new grape varieties with both the durability of native grapes and the quality of European grapes. This study focuses on the cold hardiness of Norton and Cabernet Sauvignon hybrids. Buds were collected from each hybrid once per month from December to February during the winters of 2015-2016 and 2016-217. The buds were attached to sensors and placed in a freezer. As the buds froze, heat was released. This change in energy was registered as a change in electrical signal, and data was collected by a computer and recorded in a spreadsheet. The data were then analyzed to find the temperature at which 50% of each bud was killed for each hybrid (LT_{50}) and the buds were no longer viable. The goal of this project was to find the areas of the hybrids' genome that control for cold hardiness and use that information for future grape breeding projects. The data shows that no one gene or chromosome is responsible for cold hardiness in our population; however, the study suggests that several genes may play a small role.

KEYWORDS: cold hardiness, acclimation, low temperature exotherm, norton, cabernet sauvignon, molecular breeding, quantitative trait loci

This abstract is approved as to form and content

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INTRODUCTION

Many crops have played important roles in the history of mankind, from fibrous crops like cotton to food crops like potatoes and corn. Among fruit crops, however, few other crops can compare to the grape. Grapes have played a major part in the past due to their variety of uses. Grapes can be eaten fresh, but they can also be processed into a variety of other useful products including raisins and jellies. Grape juice can be consumed on its own, but thanks to microorganisms found naturally on the grapes' skin the juice can easily be fermented to create the grape's most famous product, wine. Due to the popularity of grapes and wine, winemaking and wine culture has grown in many parts of the United States. This growth, combined with the many other valuable economic uses for grapes, has created a market for better grape varieties that produce higher quality fruit with fewer chemical costs and less maintenance.

Grape cultivation began between 6000 and 8000 years ago in the Mediterranean regions of the Near East (This et al., 2006). This led to the domestication of grapes and, in a sense, the yeast found on grape skins. Over time, grape cultivation moved into other parts of the Near East and eventually into Europe. Many of the more popular modern grape varieties, such as Pinot Noir and Cabernet Sauvignon, originated in the Burgundy and Bordeaux regions of France. The popularity of these grapes and the wines they produce has created a demand for both the plants and their wines across the globe. Traditional European grapes are believed to have been introduced to North America at two different times (Read and Gu, 2003). Spanish conquistadors planted grapes along the western coast in 1525, and English settlers introduced their varieties in the eastern

colonies in 1619 (Read and Gu, 2003.). Over the years, the North American grape industry grew considerably but not without hardships. European grapes, being adapted to certain climates and pests, were highly susceptible to damage from both environmental factors and disease. North America's diverse landscapes and climates meant that greater consideration was needed when choosing a new vineyard location. Once a vineyard was established, disease was a constant concern. One disease, caused by *Phylloxera vitifoliae*, had a large effect on the new grape industry which made establishing and maintaining vineyards difficult.

Despite these challenges, many still wanted to produce grapes commercially, and so in the 20th century farmers and researchers focusing on ways to improve grape production in the United States (Read and Gu, 2003). In the early 1900s, these improvements were mostly made through vineyard management (Read and Gu, 2003). Farmers would focus on different training and pruning methods to maximize the amount of light penetration and air circulation in the vineyard. Better light penetration and air circulation ultimately led to increased yield and less damage from disease (Read and Gu, 2003). Perhaps the biggest development during this period was the discovery that native American grape varieties could be used as rootstock for European grapes (Read and Gu, 2003). People knew that European grapes were highly susceptible to damage from *Phylloxera*; however, native grapes did not seem to be affected. It was found that damage from *Phylloxera* in these traditional grape varieties could be greatly reduced when they were grafted onto the roots of American varieties, making vineyard establishment an easier process. In the mid-1900s, grape growing began to move in a new direction. Farmers and researchers began to place more emphasis on cultivating the native

American grapes, which were already adapted to a wider variety of climates and were tolerant of the native pests, and generating new hybrid varieties between American and European grapes (Read and Gu, 2003).

Selectively growing crops in order to emphasize desirable traits and reduce the effects of undesirable traits is not, in and of itself, a new concept. Many modern staple crops of the world, such as wheat and corn, exist in their current form due to centuries of selective breeding. From this, the idea to create even better cultivars from these enhanced varieties arose. Like selective breeding, hybrid crop production is an older science. One crop variety may produce more yield than another, while the underperforming variety may be better suited to the growing environment provided by the farmer. As a result, farmers want crops that can incorporate the best aspects of multiple varieties of that same crop. Before the hybrid production process can begin, however, one must decide what traits are desirable in the crop being considered. For example, in wine grapes a superior variety would produce ample berries, each of which is resistant to common diseases while still maintaining a high-quality juice for wine making. This study looks at the hybrid offspring of two grape varieties: *Vitis aestivalis*-derived 'Norton' and *Vitis vinifera* 'Cabernet Sauvignon'. These two varieties are different in many ways, and both have positive and negative traits for the modern vineyard owner.

The first parent cultivar of our population is the Norton grape. Norton is an American grape variety grown most commonly in warm, humid areas of the American Midwest and East (Ambers, 2013). Seen frequently in states like Missouri and Kentucky, Norton grapes were first developed around 1820 in Virginia by Dr. Daniel Norton (Ambers, 2013). This variety was initially believed by Dr. Norton to be a cross between a

red ‘Bland’ variety and ‘Pinot Meunier’, both of which were growing in his vineyard. Despite the proximity of these two varieties, the resulting hybrid offspring had many traits more similar to the native grapes growing nearby than to the parents from which it is believed to have been developed. This new hybrid had a lower acid content that most American grapes, demonstrating its *V. vinifera* heritage, but the berries had an aroma much more like the native *V. labrusca* grapes and dark pigments like the native *V. cinerea* (Ambers and Ambers, 2004). This instead suggested that the Norton variety was a hybrid between a European cultivar, from which the hybrid seeds were collected, and one of the several American grape varieties found nearby. With the advent of genetic technologies in the 1900s, studies were conducted in order to determine the true ancestry of the Norton grape. DNA analyses have been conducted; however, no conclusive evidence has been found to confirm Norton’s origin (Ambers, 2013). One study used genetic markers called simple sequence repeats to identify Norton’s parents by comparing its genome to those of the “original parents”, ‘Bland’ and ‘Meunier’, as well as the genomes of many native American varieties. The study was unable to determine a precise parentage for Norton based on the data available at the time, but comparing the genetic markers available indicated that the most likely parentage for Norton is most likely a European *V. vinifera* crossed with the American species *V. aestivalis* (Stover et al., 2009).

Regardless of its origin, Norton grapes have many traits that are beneficial to farmers in states like Missouri and Virginia. Being derived from native grape species gives Norton a large advantage over its European counterparts in terms of disease, heat, and cold tolerance. As mentioned *Phylloxera* was a major problem for grape growers

during the early years of vineyard production in the Americas. This pest can severely damage the root system of non-resistant plants, leading to reduced plant health and ultimately death. This remained a major problem for any vineyard owners who were not growing adapted cultivars or did not have plants grafted onto resistant rootstocks. Norton's native background makes it tolerant of *Phylloxera* and resistant to many other diseases. These other diseases, like powdery mildew and downy mildew, are not as harmful to the plant itself but can have a large economic impact by reducing both the quality of the fruit and the yield. Because most European grape varieties are highly susceptible to these diseases, they require constant monitoring and maintenance to ensure that the damage is kept to a minimum; Norton grapes can help to save time and costs from pesticide use. In addition to its disease resistance, Norton grapes are very tolerant of the climate found throughout the Midwest. States in this region tend to have longer, more humid summers and can experience winters with highly varying temperatures. The high heat and humidity can lead to increased instances of disease, especially when plants are not properly pruned to allow proper air flow. Midwest winters can range from mild to very cold which can be a major hurdle for farmers looking to grow grapes that are adapted to consistently mild temperatures. Often, the winters in this region are not consistent. The temperature may stay well below freezing for several days, rise to well above freezing for a few days, and finally drop back down below. This can be a lethal for any grape that is intolerant of both consistent cold and rapidly changing weather conditions. Norton, as with most American grapes, was developed in this region of the United States and, as such, is well adapted to these conditions, making it an excellent choice for Midwestern farmers who want to grow wine grapes.

Though Norton has many advantages, this cultivar is not without its problems. Like many American grapes, Norton plants tend to have highly vigorous growth. These plants put a lot of resources into vegetative growth which can, in turn, take some energy away from fruit production. This is an undesirable scenario for farmers as it means extra time must be used for pruning to ensure optimal yield and quality. A major method of propagation for grape plants is through dormant cuttings which works well for many grape cultivars; however, Norton dormant cutting are slow to root, making winter propagation difficult. Furthermore, Norton plants are highly susceptible to damage from sulphur applications. Sulphur is typically used to combat powdery mildew in greenhouses, but Norton grapes will drop their leaves and potentially die when exposed to sulphur. This poses a problem when Norton shares greenhouse space with a cultivar that needs regular sulphur applications. In the field, Norton grape plants are considered to be low yielding when compared to the older, European grapes. Norton berries, in general, have smaller berries that grow in smaller clusters. As a result, more berries may be needed to produce the same amount of wine that could be obtained from a more popular variety. Another issue with Norton berries is that they are considered to be of a lower quality than those of European grapes. While Norton grapes, thanks to their hybrid nature, have a lower acid content than wild American grapes, they still have higher acid contents than more traditional wine grapes (Ambers, 2013). This higher acid concentration, paired with a lower sugar content than European wine grapes, creates a problem for Norton grape from a wine making and marketing standpoint. The different levels of sugar and acid in the berries means that Norton grape juice may need to be amended during the wine making process to achieve the desired taste. Furthermore,

Norton's American heritage gives it a unique flavor unlike those of traditional wines. This flavor is more popular in Midwestern states but has not branched out much beyond those. Norton's positive traits make it an ideal candidate for grape breeding programs; however, a suitable variety is needed that can offset Norton's negative traits. For this, the *V. vinifera* 'Cabernet Sauvignon' was chosen as the second parent during the establishment of our hybrid population.

Cabernet Sauvignon is one of the most well-known, and well liked, wine grapes in the world. As a testament to its popularity, Cabernet Sauvignon is grown around the world today. This variety originates from the Bordeaux region of France and has been grown there since the 17th century (Bowers and Meredith, 1997). Genetic studies have shown with a high degree of probability that Cabernet Sauvignon is itself a hybrid of two other French wine grapes, Cabernet franc and Sauvignon blanc. This background gives Cabernet Sauvignon many traits that are valued by growers, wine makers, and consumers alike. Grapes, as with other crops, have been selectively bred for centuries. Consequently, European grape cultivars have been developed that are well suited to their both their growing environments and the needs of the grower. Cabernet Sauvignon, like other French grapes, produces less vegetative growth than American grapes. As such, less time and energy is needed to prune and maintain vineyards, allowing farmers to focus on the areas that need the most work. Cabernet Sauvignon also roots readily from dormant cuttings under greenhouse conditions, making propagation easy and can withstand sulphur which is commonly applied in greenhouses to combat powdery mildew infections. Cabernet Sauvignon is also considered a high yielding grape variety, producing larger clusters and berries than Norton plants. This provides an advantage at

harvest time, as fewer plants can produce the same amount of yield or more. Not only are the berries of Cabernet Sauvignon larger than Norton's, but they also have a lower organic acid and higher sugar content. Fewer amendments may be needed during wine making, and the end result is a better flavored wine that is generally preferred by consumers.

Despite its many beneficial traits, Cabernet Sauvignon suffers from the same problems as other European grapes when grown in certain parts of the United States. The Bordeaux region of France has a variety of soil types but the best wines are said to be made from grapes in which the vineyards have well drained gravel soils. This region is also near the coast and contains several rivers. This gives the area an oceanic climate in which summers are usually cool and winters are cool but not cold with few extremes of temperature. Similar climates can be found in many places in the United States, especially in California, New York, and states with large bodies of water than can provide coastal effects. Missouri, unfortunately, does not have such a climate. Whereas Norton grapes grow well in the American Midwest, Cabernet Sauvignon growth can be hindered by the heavier soils and temperature fluctuations. Cold damage in particular is a major problem when growing Cabernet Sauvignon in Missouri. The cold winters can kill plant tissue all the way to the cane and, in severe cases, can result in cane death. Farmers must take special care to protect their vines from winter damage, sometimes maintaining three or four trunks per plant to replace any that do not survive. Another issue that arises when growing Cabernet Sauvignon in Missouri is pressure from disease. Thanks to American rootstocks, *Phylloxera* and other root diseases are not as bad as they once were, but other diseases like powdery mildew, downy mildew, and *Botrytis* bunch rot still cause

problems through reduced fruit quality and yield loss. Where Norton is resistant to these pests, Cabernet Sauvignon is highly susceptible; regular pesticide application is needed to ensure plant health.

From this, it should be apparent that there is a both a desire and a need for a new variety of grape that is properly adapted to Missouri's environment if Missouri is to extend its ever growing wine industry. Both Norton and Cabernet Sauvignon grapes have their advantages and disadvantages, but by creating a hybrid population between the two the impact of the negative traits may be reduced. This could allow us to produce a new cultivar that is tolerant of Missouri's climate and disease pressures while still retaining the quality and ease of propagation expected of a more traditional European grape. Through the use of molecular markers such as simple sequence repeats (SSRs) and genetic technologies, the timeline for this breeding process can be shortened significantly. Normally, a grape breeder would need to wait up to five years after every cross to test the phenotypes of their population. From there, the better plants would be maintained and new crosses performed; this process would repeat until a new cultivar was developed, a process that could take up to thirty years. The genetic tools available to modern day researchers and plant breeders allow us to easily establish a sizeable population of true hybrid offspring with only a few crosses. SSRs are especially valuable for developing true hybrid populations for a variety of reasons. First, they are PCR derived. This means that SSRs can be used to quickly 'grow' a region of interest in the target organism's DNA, and that region can then be visualized through a process such as gel electrophoresis. Second, SSRs are polymorphic, meaning that they can be different

between species. Because of this, SSRs can be used to distinguish between the parents used in hybrid development.

Tools like molecular markers allow scientists to draw connections between genetic data and the natural traits of the plants being studied. Phenotypic data can be collected and linked back to the genetic data from our population. Using this information, we can then analyze our results in an effort to locate any quantitative trait loci (QTL) within the population's genome that is responsible for the trait of interest. A QTL represents the segment of the plant's DNA that is responsible for our trait, and they can be broken into two major categories, major QTLs and minor QTLs. If a trait is controlled by a major QTL, then the effect seen is caused primarily by a single gene; on the other hand, a trait controlled by minor QTLs would be controlled by multiple genes, each of which have a small effect that leads to the overall phenotype. This study focuses on the cold hardiness aspect of our hybrid population. Cold weather tolerance is a limiting factor in the growth of any plant, and cold damage can have a large impact on the eventual yield of any crop being produced. It is important to identify any plants within our population that demonstrate high levels of cold tolerance for use in future breeding projects. In addition, this study aims to locate any QTLs associated with cold hardiness in our hybrid population.

LITERATURE REVIEW

Cold Hardiness

Winter temperatures are one of the factors that have a major impact on plant growth. A plant's ability to withstand low temperature will determine its regional distribution as well as its likelihood of survival and potential yield (Gray et al., 1997). Plants are adapted to the environments in which they were developed and will have difficulties growing in regions where winters are too severe. Tropical plants, for example, thrive in humid regions with hot summers and warmer winters, and they do not survive well in areas with shorter growing seasons and consistent cold temperatures. For plants to survive at freezing temperatures, they must be able to properly adapt to a variety of natural stimuli including day length as well as temperature (Kalberer et al., 2006). Plants adapt to seasonal changes through process called acclimation in which physical and chemical changes prepare the plant to survive an otherwise unfavorable climate. Of particular interest to this study is the process of cold acclimation, also called cold hardening. Cold acclimation can be defined as a temporary increase in a plant's ability to tolerate cold temperatures that could potentially cause cell damage.

Acclimation can be triggered by decreasing temperatures and reducing day lengths which can be detected by the plant and cause changes in gene expression, resulting in accumulation of cold tolerance related proteins (Gray et al., 1997). Acclimation manifests itself in many plant species, including grapes, in the form of dormancy during which no visible growth occurs. Deacclimation refers to reduction of hardiness levels that came from an earlier acclimation process, but can also refer to

hardiness lost due to other environmental factors like warm temperatures. Deacclimation tends to occur at a more rapid rate than acclimation, happening within a few days/weeks rather weeks/months (Kalberer et al., 2006). This is generally thought to occur because deacclimation is a less energy intensive process than acclimation, as fewer changes are needed to achieve the it (Kalberer et al. 2006). Because deacclimation can occur so quickly, recent temperatures play a strong role in the overall cold hardiness of a plant (Howell and Weiser, 1970). Warm temperatures can cause plants deacclimate, leading to damage in the event of sudden cold. Most overwintering plants can regain their cold hardiness to some degree even after brief midwinter deacclimation (Repo, 1991); however, the ability of a plant to reacclimate is decreased as the exposure to deacclimating factors increases (Kalberer et al., 2006).

In grapes, the level of cold hardiness is primarily dependent on the species being considered. Some varieties are very sensitive to cold and will suffer damage in temperatures as high as 28 °F while the hardiest varieties, such as the native *V. riparia*, can survive down to -40 °F (Howell, 2000). Winter injury accounts for large economic losses in grape production, and one good freeze can potentially wipe out a year's crop if proper precautions were not taken (Zabadal et al., 2007). Even if the plant survives winter, cold damage can leave the plants more open to bacterial infection in the coming season (Davenport et al., 2008) which can reduce later wine quality, furthering the economic impact of winter (Zabadal et al, 2007).

Cold acclimation in grapes occurs in two main stages (Zabadal et al., 2007). The first stage occurs in early fall. During this time, American grapes begin acclimation in response primarily to shortening day lengths (Wolpert and Howell, 1985) while European

varieties respond to both shorter days and lowering temperatures (Fennel, 1992). Temperatures during the first stage are generally low but not freezing; thus, plants do not achieve full hardiness at this time (Zabadal et al., 2007). The second stage of grape cold acclimation occurs when temperatures begin to consistently drop below freezing. This is the period during which grapes will drop most of their leaves (Zabadal et al., 2007). Dramatic increase in cold hardiness can be seen at this stage, with hardiness increasing as the temperature continues to remain below freezing (Hamman et al., 1996). Grapes will achieve maximum cold hardiness in this second stage of acclimation, but temperature fluctuations during midwinter months can cause grape vines to deacclimate quickly, especially after the bud chilling requirements have been met (Odneal, 1984). The rate at which a grape vine will acclimate or deacclimate is largely dependent on species. For example, the native Concord grape, *Vitis labrusca*, will lose and gain hardiness more rapidly than a *vinifera* grape like Cabernet Sauvignon (Wolf and Cook, 1992).

There are several systems in place to measure cold hardiness. Some prefer to use visual field evaluations after low temperature events (Burke et al., 1976) while others simulate freeze event in lab conditions. One example of using field evaluations can be seen when Dami et al., 2012, performed an assessment of bud cold hardiness in Ohio after a freeze event in 2009. This group performed a visual evaluation of buds from vineyards that had experienced heavy winter damage, and they used a simple scale to determine bud health. Buds with brown or black cross sections were considered injured while healthy buds were green. In addition, the percent injury for each bud was calculated. This information was used to aid the researchers in determining the most appropriate pruning system to promote vine regeneration after winter freeze events;

however, they concluded that pruning had no physiological impact after extensive winter injury (Dami et al., 2012). Another system of measuring cold hardiness is the use of a system called differential thermal analysis (DTA). As plants prepare for winter, physiological changes take place allowing the buds to supercool (Mills et al., 2006). Supercooled water freezing outside of plant cells releases heat which is referred to as the high temperature exotherm; supercooled water freezing in the plant cells is called the low temperature exotherm (LTE) (Mills et al., 2006). It is this intracellular freezing which causes the most damage to the plant and can be lethal (Burke et al., 1976). Mills et al., 2006, used a DTA system which measured the change in heat released while buds were subjected to a consistently lowering temperature. The researchers used this system to find the lethal freezing temperatures of the buds from several cultivars and to test the effect of surface moisture on bud hardiness. It was found that freezing events occurring in the -5 to -10 °C range were associated with nonlethal freezing of extracellular water. The researchers also demonstrated that buds with high levels of surface moisture were susceptible to cold damage at warmer temperatures than their dry counterparts (Mills et al., 2006).

Studies on cold hardiness have been conducted for both the parent cultivars of our population. Lipe et al., 1992, performed a DTA study of Chardonnay and Cabernet Sauvignon bud deacclimation when evaporative cooling was used. The vineyards in question were located on the Texas High Plains. In this area, freeze injury occurs more often before budbreak for Cabernet Sauvignon. This happens because Texas may experience period of warm winter weather, resulting in loss of hardiness in Cabernet Sauvignon plants. The newly deacclimated vines are then more susceptible to damage

from frost and sudden cold weather events. The researchers used DTA to determine the cold hardiness of buds under normal growing conditions in the area as well as the hardiness of buds that were kept cooler during winter through the use of microjet sprinklers and evaporative cooling. This method was able to successfully protect the vines from early deacclimation, thus reducing the risk of cold damage near budreak (Lipe et al., 1992).

A study was conducted by Gu et al., 2001, to determine the rates of acclimation and deacclimation of bud hardiness in Norton, Vignoles, and St. Vincent grapes in Mountain Grove, Missouri. Once again, this study used a DTA system to simulate cold events and measure the results. The bud hardiness was first measured after a period of acclimation during which the canes were held at a constant -10 °C. Later buds were then deacclimated through storage at a constant 20 °C. It was found that the maximum hardiness for each cultivar was dependent on the amount of time the plants were given to adapt to the new temperature (Gu et al., 2001). The longer plants were held at a sub-freezing temperature, the more hardy they became, and canes subjected to above freezing temperatures became less hardy as time went on (Gu et al., 2001). Among the tested varieties, Norton was found to be the most cold hardy. In addition, each variety reached a level of peak hardiness that did not increase despite continued acclimation time, suggesting that cultivars have a predetermined maximum hardiness that can be reached in a given season (Gu et al., 2001).

Factors Influencing Cold Hardiness

As with most traits, there are many factors that can influence cold hardiness, but these can be broken into two major groups: environmental factors and genetic factors. As mentioned, cold acclimation is triggered initially by seasonal changes in temperature and day length; however, the actual level of hardiness attainable can be affected by many aspects of both the cultivar being grown and its environment. Studies have shown that vineyard site selection is important in determining the overall hardiness of a grape plant. Locations with poorer air drainage were, in general, consistently colder. As a result, grapes grown in these environments acclimated faster and achieved a greater degree of cold hardiness than grapes grown on a site with good air drainage (Stergios and Howell, 1977). Knowing about air drainage in a potential vineyard site can be crucial when choosing a variety to plant, as the origin of a variety impacts the level of cold that can be tolerated. Data has shown that European grapes, which were developed in milder climates, tend to be less hardy, while varieties that developed in regions with less consistent climates could achieve a greater peak hardiness (Ferguson et al., 2013). Researchers in Texas studied the effects of regulated deficit irrigation on Cabernet Sauvignon and how this relates to acclimation. Basinger and Hellman used deficit irrigation in an attempt to induce earlier acclimation of the test vines. They found that deficit irrigation did improve water-use efficiency in the grape vines; no negative effects were seen on the yield, fruit quality, or cold hardiness, but neither was there an improvement in primary bud hardiness (Basinger and Hellman, 2006). This result was consistent with the outcome a general irrigation study conducted by Hamman and Dami in 2000.

Another set of studies observed the effect of photoperiod and its role in cold acclimation. Fennel and Hoover in 1991 tested two American grape varieties to determine whether or not photoperiod was a primary trigger of cold acclimation. This was accomplished by raising the vines at a constant temperatures and exposing groups to differing amounts of light ranging from 15 hours to 12 hours. The results showed that reduced photo period led to reduce cane growth rates and cause a small change of 2-3 °C in the tested vines (Fennell and Hoover, 1991). They speculate that, while the increase in hardiness may be small, this could promote earlier, more rapid cold acclimation (Fennell and Hoover, 1991). A 1993 study conducted by Huner et al. aimed to learn why reduced photoperiod and temperatures prompts acclimation by measuring the photosynthetic rates of cold tolerant plants. This study was not specifically focused on grapes but cold hardy plants in general. The scientists found that during times of reduced photoperiod and temperature down regulation of phototsystem II could be seen in cold tolerant plants. This suggested that cold hardiness of any plant species is dependent mostly on the mechanisms that plant uses to survive freezing temperatures, but there is a possible link between reduced photosynthetic rates causes by short days and cold weather and the onset of cold acclimation (Huner et al., 1993).

Over the years, many studies have been conducted on the many ways to research cold hardiness as well as ways that both environmental and cultural elements will influence it. It has been shown that buds taken from different areas of the grape vine canopy can display different levels of cold hardiness, with the difference in hardiness between primary buds on the same plant being as great as 12 °C; however, the researchers claim that this extreme difference is likely to the unseasonably warm weather

present just before testing (Howell and Shaulis, 1980). A midwinter pruning study conducted in Washington found that cane pruning did not seem to cause earlier budbreak in Cabernet Sauvignon and did not have a noticeable impact on cold hardiness, though this study only accounts for one season (Wample, 1994). Research on the effect of rootstocks found that the rootstock used did not have much effect on the in-canopy distribution of canes with superior hardiness, suggesting that any increase or decrease in hardiness arises from rootstocks was secondary in nature (Striegler and Howell, 1991). Likewise, studies on harvest date have shown that time of grape harvest does not alter the bud cold hardiness of dormant *V. vinifera* species, and that any perceived effect is likely dur to variation in that year's climate rather than the harvest date itself (Wample and Bary, 1992). Of greater interest in recent years is a study on the effects of Japanese beetle defoliation in Norton and Cabernet Sauvignon grapes. The researchers created groups of test plants and performed different spray regiments on each. It was found that Japanese beetle defoliation, when left unchecked, greatly reduced the bud cold hardiness for both cultivars, but a weekly spray was effective in mitigating this damage (Hammons et al., 2010).

These previous studies focused mainly on the way in which grape plants interact with their environment and how this changes the hardiness of the plant. There have been, however, many studies concerning the changes that grapes undergo on a cellular level as acclimation occurs. One study examined the sugar composition of grape buds during cold acclimation and deacclimation. It was found that concentrations of glucose, fructose, raffinose, and stachyose increased as the cold hardiness increased and began to decrease as deacclimation began, suggesting that these sugars and their associated pathways may

play a role in bud hardiness (Hamman et al., 1996). A Chinese study also found that cold hardy grapes had higher sugar concentrations as well as a higher level of abscisic acid than non-tolerant varieties (Ma et al., 2010). Other studies have found that proteins may accumulate as well as sugar and abscisic acid in cold hardy grapes. The presence of late embryogenesis-abundant (LEA) proteins has been proposed as a possible means of enhancing cold acclimation; there are several groups of these proteins, but all of them play a role in cold hardiness. The 47kD LEA proteins, while they are related to plant dormancy, do not seem to affect cold acclimation; however, the 27kD LEA-like proteins only showed accumulation in cold acclimated buds (Salzman et al., 1996). Accumulation of these proteins and sugars assists grape plants in achieving peak cold hardiness, but evidence also suggests that enzymes within grapes undergo changes during cold acclimation (Guy, 1990). These changes allow the enzymes to function better and remain more stable and active at lower temperatures (Guy, 1990). Enzymes are not the only part of the cell to undergo changes during cold acclimation. In grapes, accumulation of abscisic acid and various proteins (both structural and otherwise) can lead to increased cell strength during cold acclimation, preventing cell rupture and collapse during a freeze event (Rajashekar and Lafta, 1996).

Plant Breeding and Molecular Technologies

Grape vines can live for many years in a vineyard without needing to be replaced. This makes grapes an enticing crop for potential growers as the initial planting can provide years of income. This longevity does, however, can make breeding grapes for cultivar development a lengthy and difficult task. Traditionally, cultivar development was

performed by using the pollen from one parent on the flowers of another parent and testing the offspring for desirable traits. This process would continue until a new variety had been cultivated that demonstrated the traits being studied. In annual crops, this process can be repeated multiple times a year, allowing for rapid development. In perennial crops like grape, this is not the case. As with any crops, the cross must first be made, and a standard procedure has been developed that demonstrates this (Reisch, 2001). First, pollen is collected by removing flowering clusters from the male parent, drying the clusters for 1-2 days, and shaking the clusters to remove the pollen. While the pollen is drying, the female flowers are prepared to receive the pollen by removing the flower cap and any anthers, thus preventing self-pollination in perfect flowered varieties. Once done, the female clusters are covered to prevent wind pollination and allowed to sit for 1-3 days. After this time, the pollen is applied to the female flowers which are then covered again. Berry development in the crosses is monitored, and seeds are collected at harvest time. From there, seeds can be germinated and planted for use in future research. Some traits can be measured while the plants are still young, such as sulphur sensitivity. Grapes grow for 3-5 years before producing fruit, so any berry related research must wait until then. From this it can be seen that traditional grape breeding is a time-consuming process and can take up to 30 years. Fortunately, the advent of molecular technologies has allowed researchers to speed up this process make more accurate crosses. By sequencing the DNA of potential parents of a plant breeding program, differences can be detected between varieties using DNA sequences known as markers. These markers can be detected using the polymerase chain reaction (PCR) with primers specific to the species being studied, and DNA markers can even be used to successfully differentiate

between multiple varieties of the same species (Striem, et al., 1994). One major type of marker used in such cultivar studies is called the simple sequence repeat (SSR). SSRs are short, repeating DNA sequences of 2-5 base pairs that are found in the genomes of all organisms (Collard and Mackill, 2008). Due to their repetitive nature, SSRs are more likely to mutate and the sequence become longer or shorter through a process called strand slippage. These mutations make SSRs highly polymorphic even among organisms of the same species (Collard and Mackill, 2008). Another type of maker that has become important in genetic studies is the single nucleotide polymorphism (SNP). SNPs are changes to a single nucleotide at a given point in the genome and can also be used to distinguish between individuals.

These markers are important to the modern plant breeder for a variety of reasons. First, markers can be developed that distinguish between the parents of a hybrid population. This allows researchers to use those same markers to determine which of the offspring are true interspecific hybrids. Interspecific hybrids contain two alleles for each gene, one from each parent. Identifying interspecific hybrids can be done while the plants are still very young; a huge benefit for plant breeders working with long lived crops that require many acres to grow. Rather than planting all offspring from a cross, breeders can use these tools to identify and plant only the true hybrid plants, saving time and space.

Another valuable use of genetic markers is the role they play in construction of a genetic map. Once a population has been established, marker data can be used to create a map of the genome for that variety (Staub et al., 1996). This process involves several steps, but the basic requirements are a mapping population, calculations of recombination frequencies using this population, establishment of linkage groups, and determination of

map order (Staub et al., 1996). With all of this information, researchers can then begin to combine the genetic data with physical that has been collected. The goal of this data combination is to identify the presence of any quantitative trait loci (QTL) in the genome of the test population. A QTL represent a portion of the genome that is responsible, either primarily or partially, for the physical trait being studied. Once a QTL has been identified, that information can be used in future to enhance and speed up plant breeding projects (Asins, 2002). If a researcher knows the which genes are responsible for a trait, such as cold hardiness or disease resistance, and they know which markers are associated with those genes, plants can be screened at a very young age to determine which ones have the desired trait. In the case of grapes, this saves the researcher the years that would otherwise be lost to growing vines that may or may not display the trait. One method of identifying QTLs is through the use of SNPs and a program called TASSES (Trait Analysis by aSSociation, Evolution, and Linkage). This program can analyze data using a variety of models and will even display results in a graphical format (Bradbury et al., 2007). TASSEL is capable of running on less powerful computers than other software but can easily handle both large and small studies (Glaubitz et al., 2014). This program makes QTL identification a relatively simple process, which is a great help to plant breeders. Although QTLs are mainly used in breeding, there are other areas where they could prove useful by aiding in the study of complex traits, like disease resistance, and how those traits are connected to protein production and genetic regulation (Asins, 2002).

MATERIALS AND METHODS

Test Population

A hybrid population between *Vitis aestivalis*-derived 'Norton' and *Vitis vinifera* 'Cabernet Sauvignon' was initially developed in 2005 resulting in 98 individuals at Missouri State University's Mountain Grove Fruit Experiment Station. Of these individuals, 74 were produced by crossing a female Norton with a male Cabernet Sauvignon, and the remaining 24 were the product of a reciprocal cross (male Norton x female Cabernet Sauvignon) (Adhikari,et al., 2014). This population was expanded in 2011 by an additional 180 genotypes using the procedure described by Adhikari et al. (2014). Pollen from the male parent, Cabernet Sauvignon, was collected and dried overnight under a 60W lamp. The female parent, Norton, was emasculated prior to pollination, and the exposed clusters were covered with paper bags to prevent unwanted pollination. Cabernet Sauvignon pollen was applied the following morning using a small brush; the bags were then placed back on the clusters. Seeds were extracted after harvest and stored in sterilized sand at 4 °C for 3 months. Seeds were then planted and allowed to germinate in a greenhouse. DNA was extracted from the seedlings using Qiagen DNeasy Plant Mini Kits according to the manufacturer's protocol. This process involved freezing and grinding 100mg of leaf material using liquid nitrogen, lysing the cells, and removing unwanted cell components via Qiagen's supplied DNA extraction columns. PCR was performed on the DNA for each seedling using three primers per reaction in addition to a WellRED labeled M13 sequence. The thermal cycler was set to run a touchdown protocol in which the initial 64 °C annealing temperature dropped 1 °C each cycle for ten cycles.

PCR products were visualized using gel electrophoresis. Hybrid screening and analysis was performed using a GenomeLab GeXP capillary sequencer and analysis software (Beckman Coulter, Inc., Brea CA).

Equipment

Data was collected using system called differential thermal analysis (DTA). This type of system uses plant material attached to thermoelectric modules (TEM) which are then placed in a freezer. DTA functions by measuring the heat of fusion released when supercooled water in the test material freezes (Wample et al., 1990). The collected data can then be expressed as the low temperature exotherm (LTE) for a given sample. LTE is the temperature at which supercooled water in the sample has frozen and represents a lethal temperature for the organism being studied (Wample et al., 1990). A freezer located at MSU's Fruit Research station was fitted with a Watlow Series 942 (Watlow Electric Manufacturing, St. Louis, Mo) microprocessor-based ramping controller. This a programmable controller that must be operated separately of the computer and is capable of both heating and cooling programs. A removable sensor unit consisting of 30 thermoelectric modules (Melcor model CP1.4-127-045L) was constructed by Bill Agee at Missouri State University in 2015 using old parts from a previous cold hardiness study. The sensors were built into a sealable box the prevented outside factors from affecting the sensors. A 32 channel system was used, allowing two temperature modules in addition to 30 TEMs for samples. Data was recorded with a custom program created using National Instruments LabVIEW system design software (Agee, 2015). The sensors collect data by recording the difference in temperature between the two sides of each TEM as a voltage.

The voltage magnitude is proportional to this temperature difference. Support circuitry was required to properly read, magnify, and record the signal from the TEMs (Agee, 2015). Live graphs for both temperature and voltage data were shown in the program; however, data from each individual sensor was saved into its own column in a spread sheet for later analysis.

Preliminary Research and Test

At the end of November 2015, a field evaluation of each genotype was performed to determine which plants were healthy enough to be included in the study. Plants were evaluated using the three following criteria: age (plants newly planted or not yet reaching the high wire of the trellis were not sampled), green tissue availability (some plants already had too much dead tissue to make them viable), and amount of material. Plants were chosen so that sampling would not interfere with other student projects plant health later in the growing season. To evaluate, canes were trimmed from the plant to determine damage and bud availability. It was decided that 144 plants, 44 from the 2005 population and 100 from the 2011 population, had sufficient material for the study. This evaluation was performed again in fall of 2016, and this time a total of 165 individuals were used.

Research into the average bud hardiness of both parent cultivars was conducted to assist in designing the best program for the freezer. Data from Washington State University showed that Cabernet Sauvignon had an average LT_{50} of -13 °C from September to March, with the lowest LT_{50} of -24 °C occurring in early January (WSU Research and Extension, 2015). Previous research has shown Norton buds to be hardy to temperatures as low as -28 °C (Gu, et al., 2001). The controller was programmed based

off of this information and the procedure described by Mills et al. (2006). The controller was set to hold the freezer at 4 °C for 1 hour before dropping to -10 °C over the course of 1 hour. From there the freezer was taken down to -40 °C at a cooling rate of 2 °C per hour. The freezer was then held at -40 °C for 1 hour. Once the program was set up, a test run was conducted using only buds from the parent cultivars. The test consisted of the following experimental designs: 1) buds removed from the cane at the base and placed on the sensors cut side up 2) buds removed from the cane at the base and placed on the sensors cut side down 3) buds removed from the cane leaving 2mm of material and placed cut side up 4) buds removed from the cane leaving 2mm of material and placed cut side up. Methods 1 and 2 showed an LT_{50} much lower than expected, likely because cane tissue is needed for the buds to supercool. Methods 3 and 4 showed LTE values closer to what was expected; however, the peaks from method 4 were sharper. From this, method 4 was chosen for the main experiment. Because the sensor unit can only accommodate 30 samples, additional tests were conducted to determine the effects of storage on cold hardiness. Fresh buds were collected and analyzed alongside buds that had been stored at 4 °C overnight. It was found that storing the buds overnight did not have a significant impact on the overall hardiness of the buds.

Bud Collection, Storage, and Analysis

Buds were collected from each of the 144 genotypes at three points during the winter 2015-2016 season (December, January, and February) and took place over the course of one week (due to equipment limitations) during each of these months. Eight to ten primary buds were chosen from each plant. Buds were left on the cane and stored at 4

°C overnight (12 hours). This was done so that all buds would be at the same starting temperature for the experiment despite the varying temperatures throughout the weeklong test period. The next morning, buds were removed from the cane using a razorblade such that 2mm of cane material was left to allow super cooling. Each genotype was given one sensor in the unit, and 8 buds from a given genotype was placed onto a single sensor with the cut side facing up. The buds were held to the sensor using parafilm, and the sensor unit was placed into a plastic freezing chamber. This freezing chamber was sealed and placed in the freezer after which the program was started. As the freezer was running, the next set of 30 sample was collected and stored at 4 °C. Once the freezer had completed its program, a spreadsheet containing all the data was saved. This process was repeated until all 144 genotypes had been sampled for that month. The same procedure was used for the 165 genotypes of the 2016-2017 season. The collected data was plotted into a line graph in which peaks represented the change in voltage registered by the sensor at a given temperature. The peaks for each of the eight buds would form clustered areas, and peaks that were extreme in either direction (+/- 3 °C of the main cluster) were discarded as being erroneous, likely as a result of smaller, secondary buds or cane tissue ending up on the sensor. The graph was used to find the freezing temperature for each individual bud of a given genotype, and the average freezing temperature of each plant calculated. This was done by taking the temperature at which each peak occurred in the graph of a given plant. The values for each peak would be averaged to provide the overall average hardiness of that plant. The average LTE data for each genotype was incorporated into genetic data that was developed in collaboration with Cornell University through the VitisGene project; theis genetic data consisted of 43,000 individual single nucleotide

polymorphisms (SNPs). The combination genotype/phenotype data was analyzed using TASSEL (Trait Analysis by Association, Evolution, and Linkage, Buckler Lab) software and RQTL in order to identify any major quantitative trait loci associated with cold hardiness. TASSEL is able to generate a kinship map for our population using the marker data developed by Cornell. By linking the genetic data, the kinship map, and the phenotype data, TASSEL can generate a Manhattan plot using our populations SNPs. This plot displays the logarithm of odds (LOD) for each chromosome in our population. To identify a major QTL, a strong peak with a minimum LOD of 3 is desired. An LOD of 3 represents 1000 to 1 odds that the observed results are not due to chance.

RESULTS

Year One

The change in electrical signal as the buds froze was recorded, the values of which were then graphed. This showed the freezing temperature for each bud from each genotype (Figure 1). The average lethal temperature (LT_{50}), the point at which 50% of the bud is damaged and the bud is no longer viable, was found by averaging the LT_{50} of all tested buds from each plant. Norton was the hardiest plant in the study with an LT_{50} of -29.2 °C. Cabernet Sauvignon had an LT_{50} of -20 °C. The hybrids showed segregation for cold hardiness, typically staying between the parent values (Figure 2). The least hardy hybrid was NxCS 146 with an LT_{50} of -19.9 °C. The hardiest hybrid was NxCS 147 with an LT_{50} of -25.5 °C. The average LT_{50} values for each genotype are displayed alongside average parent data in Figure 2. Samples numbered above 100 (102-250) are from hybrids planted in 2011; samples numbered below 100 (1-79) are from hybrids planted in 2005.

Year Two

Data in year two was collected and analyzed in the same way as year one. In general, the LT_{50} values were lower than in the previous year though exceptions are present. The average LT_{50} of Norton was -26.7 °C, and the average of Cabernet Sauvignon was -19 °C. Again, the hybrid offspring showed segregation, typically within this range. The least hardy hybrid was sample 162 with an LT_{50} of -20.2 °C; the hardiest

hybrid was sample 126 with an LT_{50} of -24.9 °C. The collected hardiness data of all samples alongside the average parent values for the second year are shown in Figure 3.

Genetic Analysis

Cold hardiness data was joined with genetic data using the program TASSEL and used to generate a Manhattan plot (Figure 4). The plot shows the likelihood of a gene controlling cold hardiness. Each colored bar on a Manhattan plot represents one chromosome (in this case Norton chromosomes 1-19). Bars with a strong, consistent peak displaying a logarithm of odds (LOD) greater than three would indicate the presence of a major QTL on that chromosome. No such peaks are seen in this data; however, a potential peak is seen on Norton's chromosome 10, which could mean there is a minor QTL present. Data was also analyzed using the R/QTL programming package to further confirm this result (Figure 5).

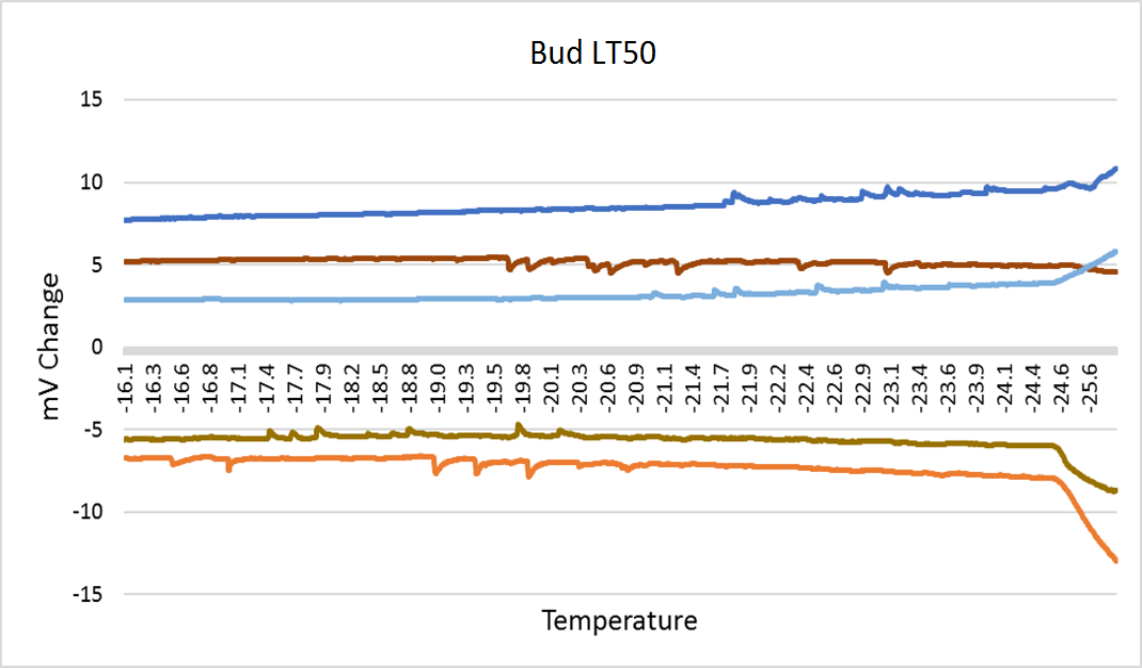

Figure 1: Example DTA Data – Each line represents one genotype (individual plant) from our population. Each peak on the line represents the lethal freezing temperature for a single bud from that plant. For each genotype, an average value from eight buds for each genotype was taken (i.e. eight peaks from each line).

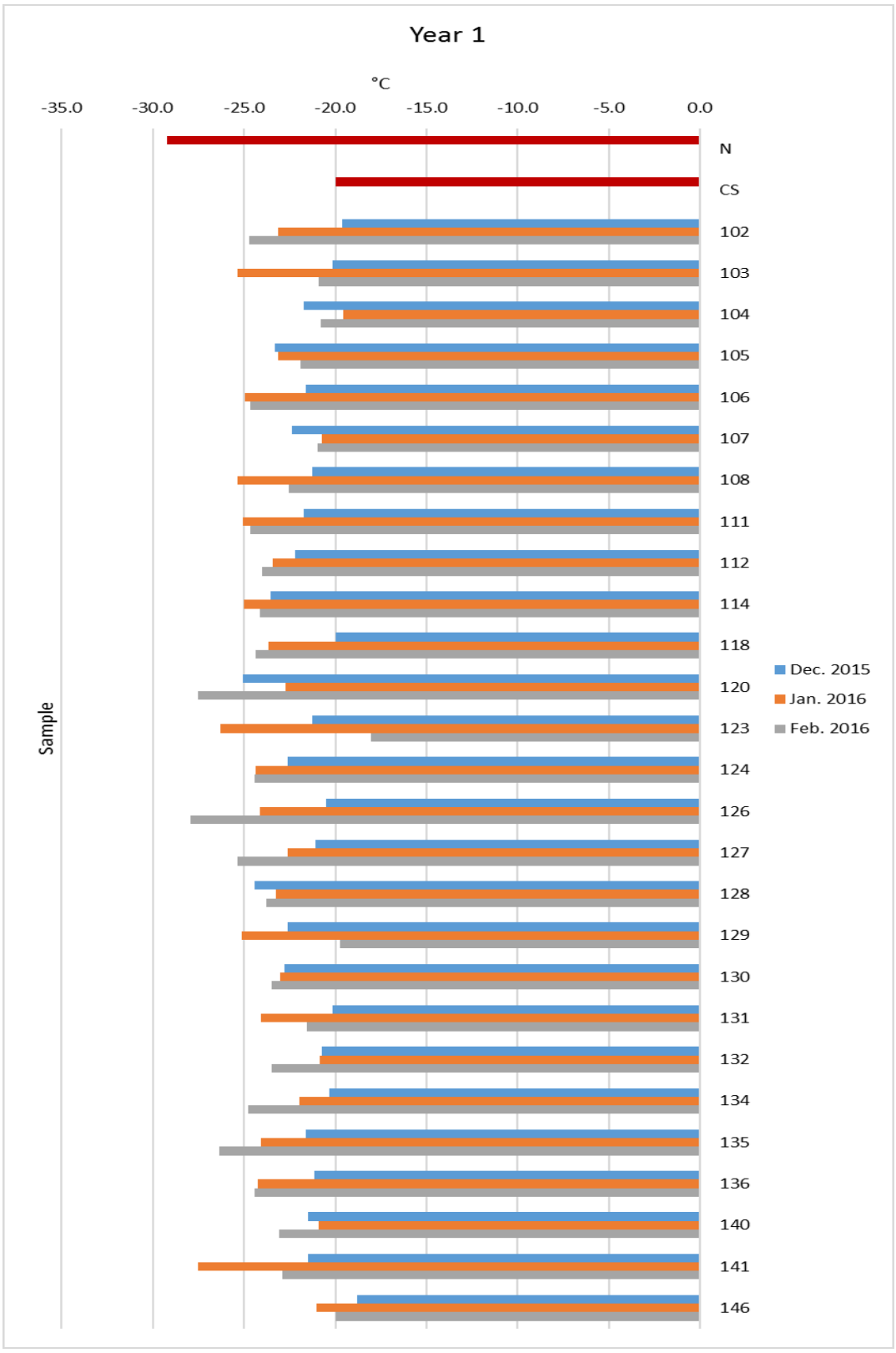

Figure 2: Year 1 (2015-2016) Data. This chart shows the average lethal freezing temperature of samples 102-146 within our population for each month of the study alongside the average lethal temperature for the parents.

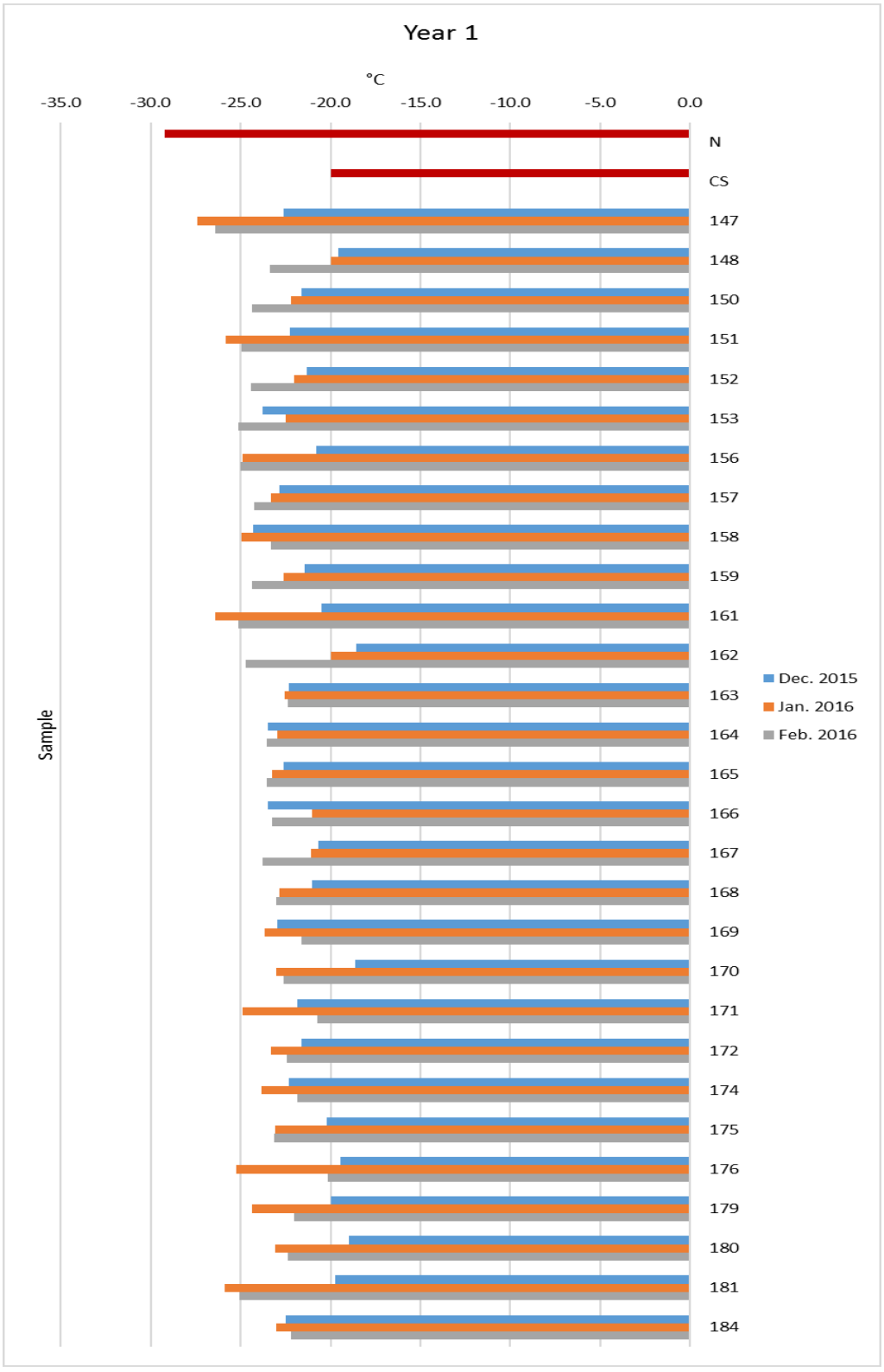

Figure 2 (cont.): Year 1 (2015-2016) Data. This chart shows the average lethal freezing temperature of samples 147-184 within our population for each month of the study alongside the average lethal temperature for the parents.

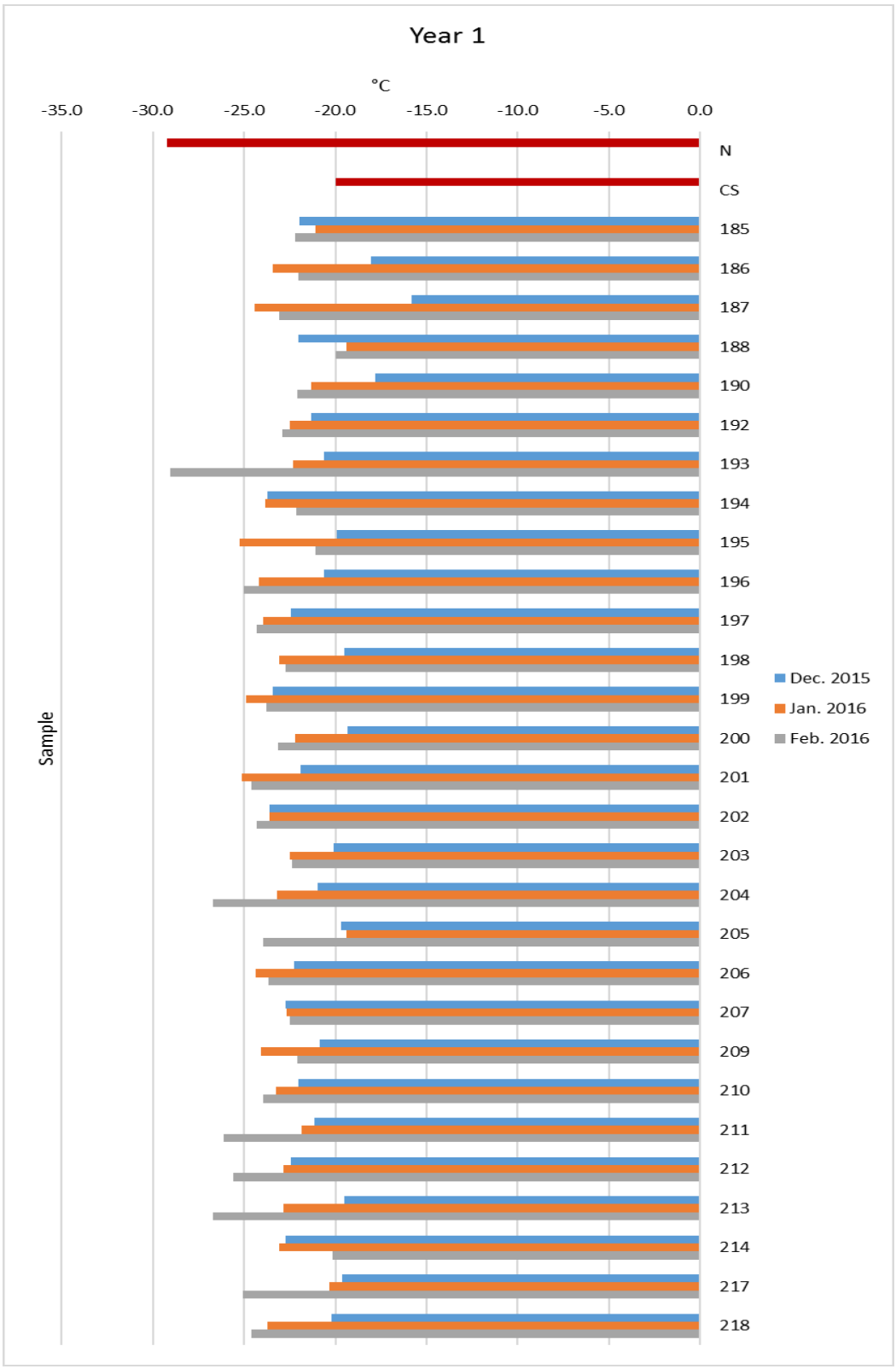

Figure 2 (cont.): Year 1 (2015-2016) Data. This chart shows the average lethal freezing temperature of samples 185-218 within our population for each month of the study alongside the average lethal temperature for the parents.

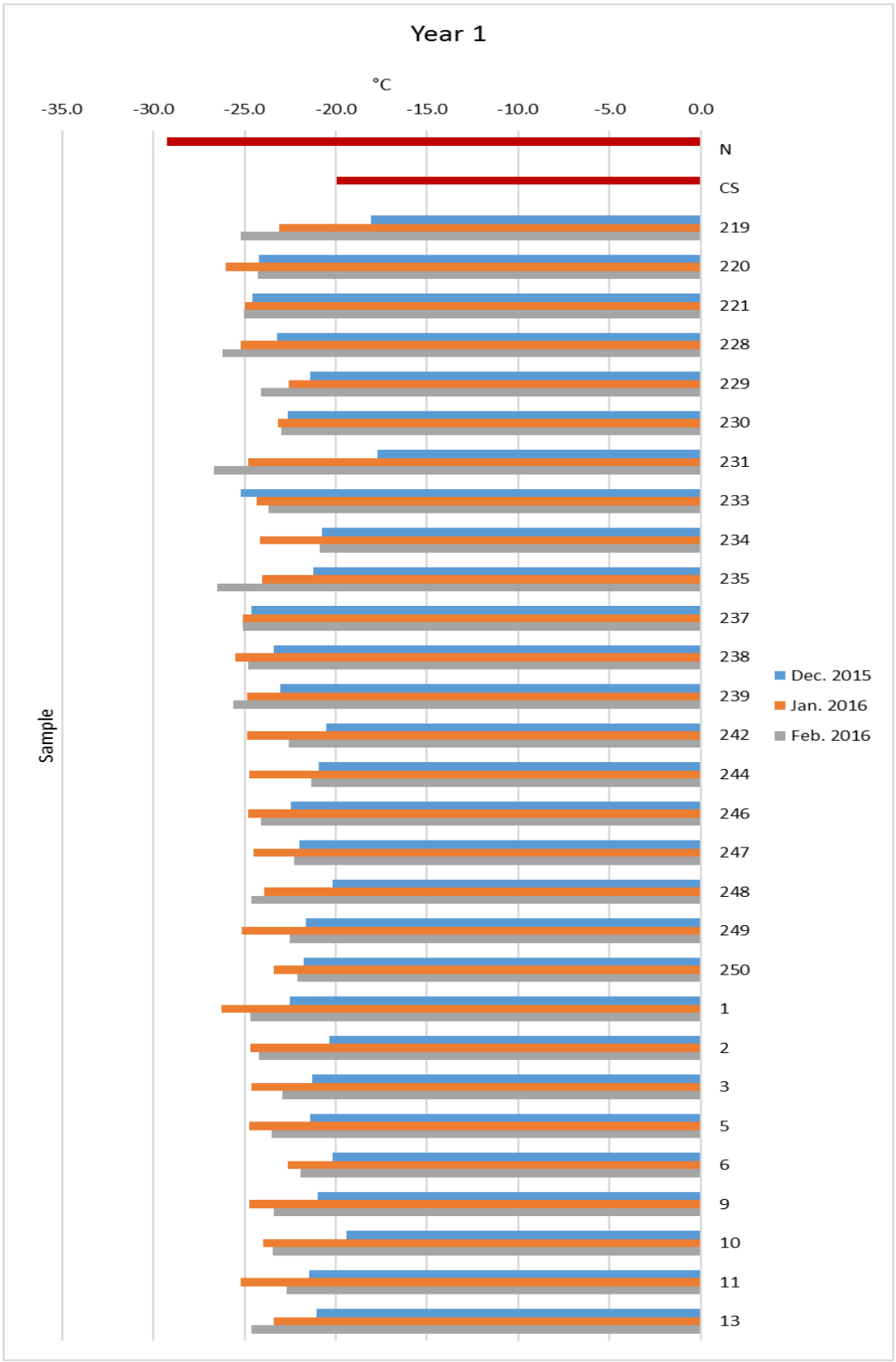

Figure 2 (cont.): Year 1 (2015-2016) Data. This chart shows the average lethal freezing temperature of samples 219-13 within our population for each month of the study alongside the average lethal temperature for the parents. Sample numbers below 100 represent plants from the 2005 population.

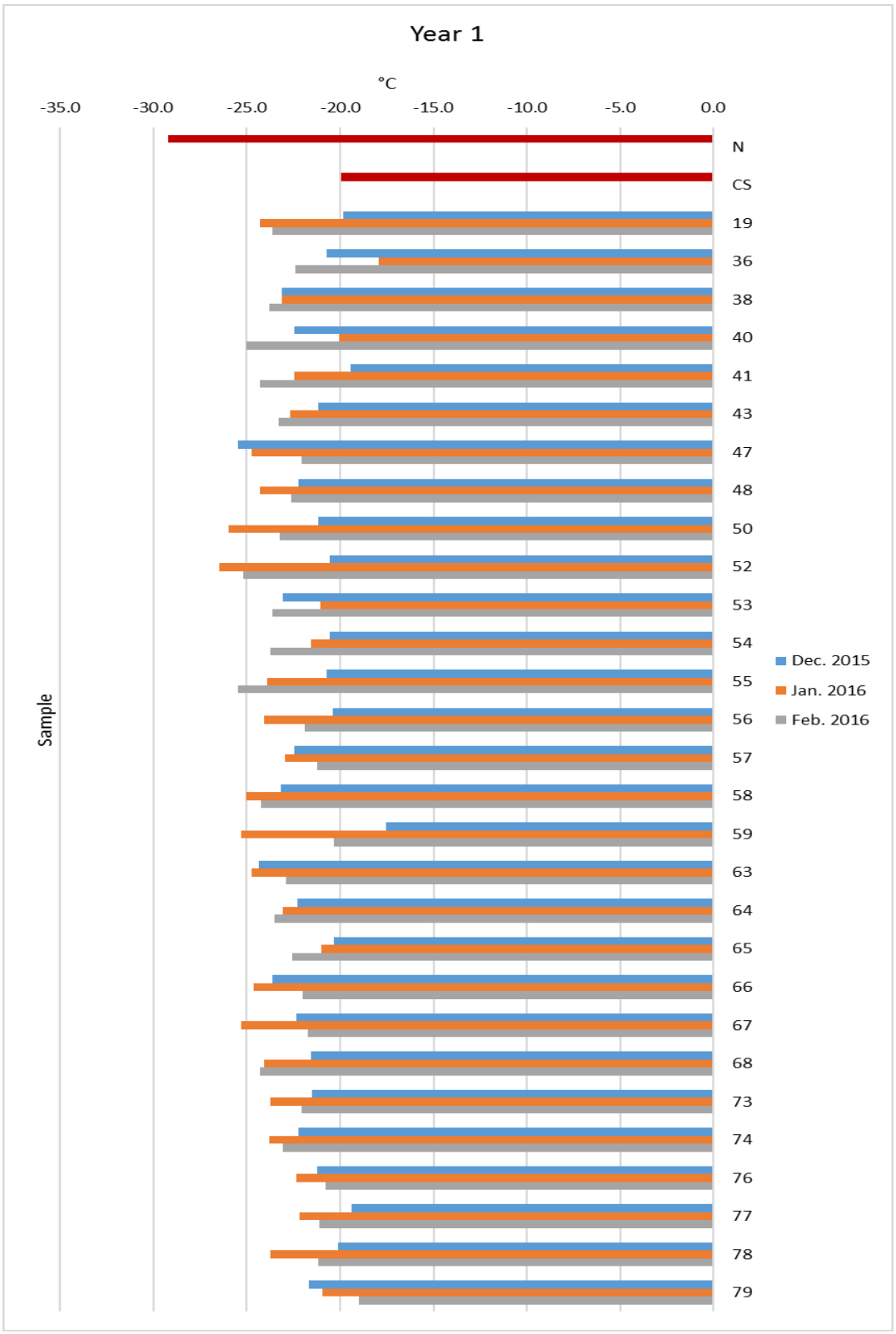

Figure 2 (cont.): Year 1 (2015-2016) Data. This chart shows the average lethal freezing temperature of samples 19-79 (2005 population) within our population for each month of the study alongside the average lethal temperature for the parents.

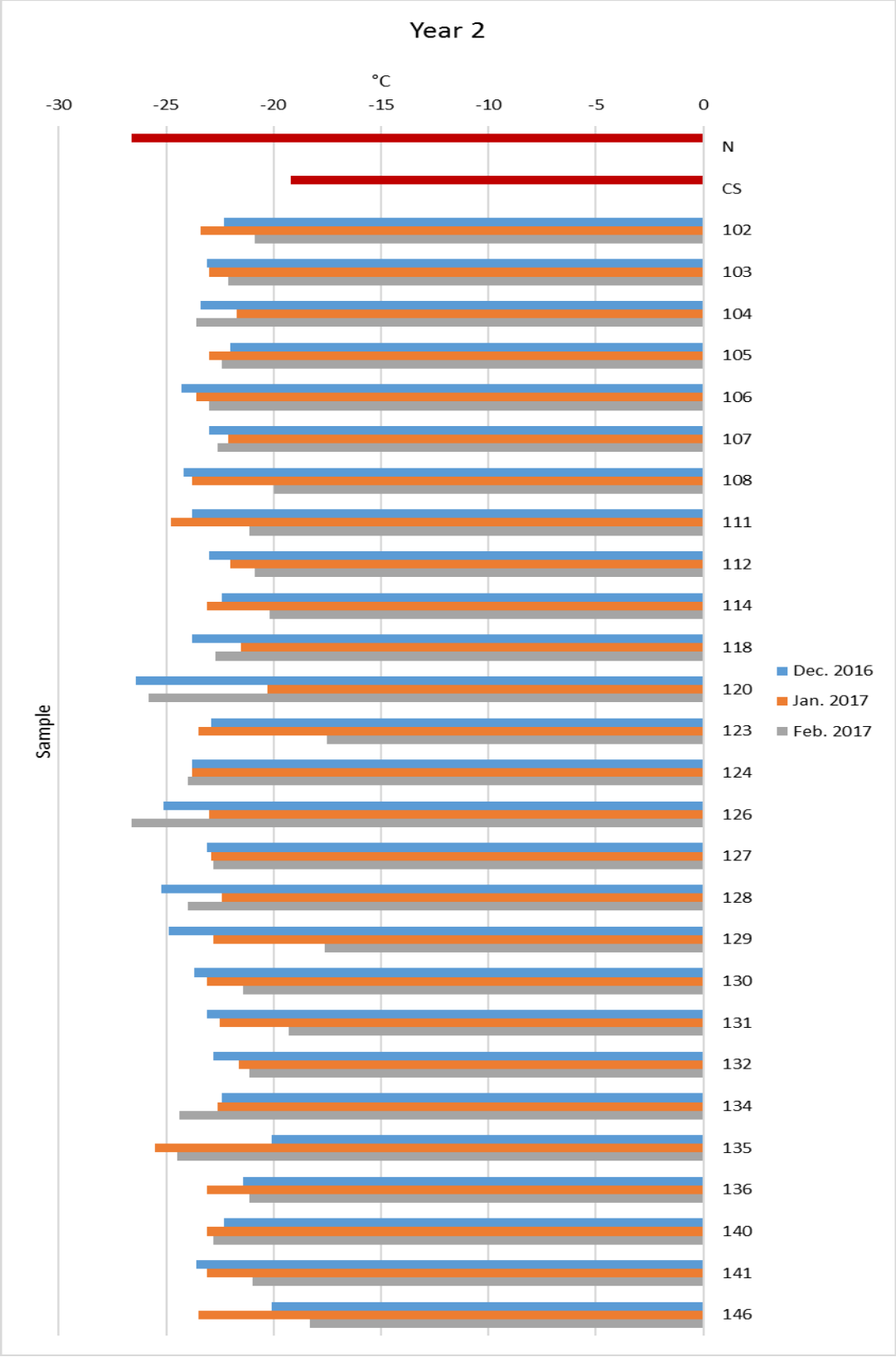

Figure 3: Year 2 (2016-2017) Data. This chart shows the average lethal freezing temperature of samples 102-146 within our population for each month of the study alongside the average lethal temperature for the parents.

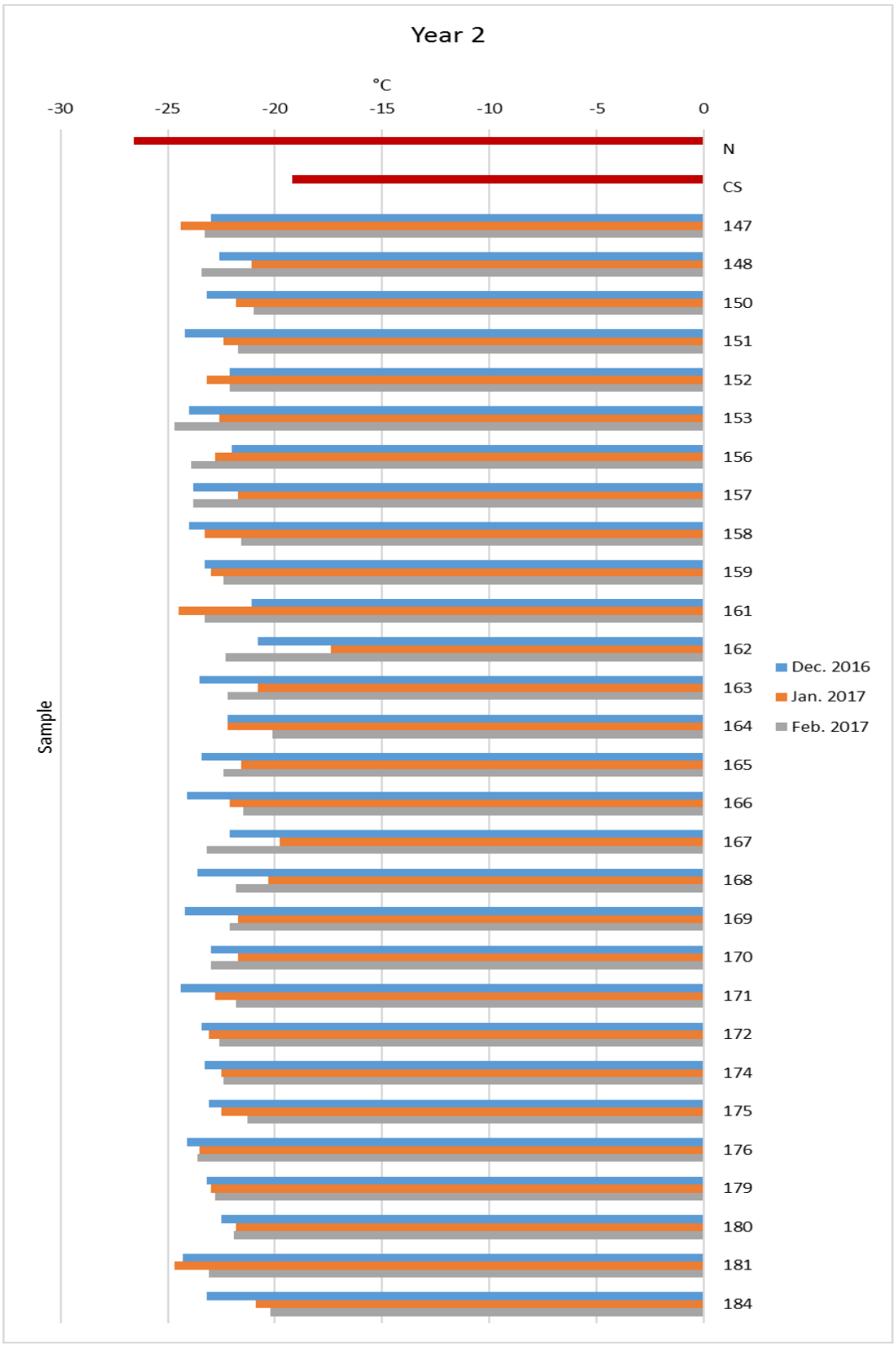

Figure 3 (cont.): Year 2 (2016-2017) Data. This chart shows the average lethal freezing temperature of samples 147-184 within our population for each month of the study alongside the average lethal temperature for the parents.

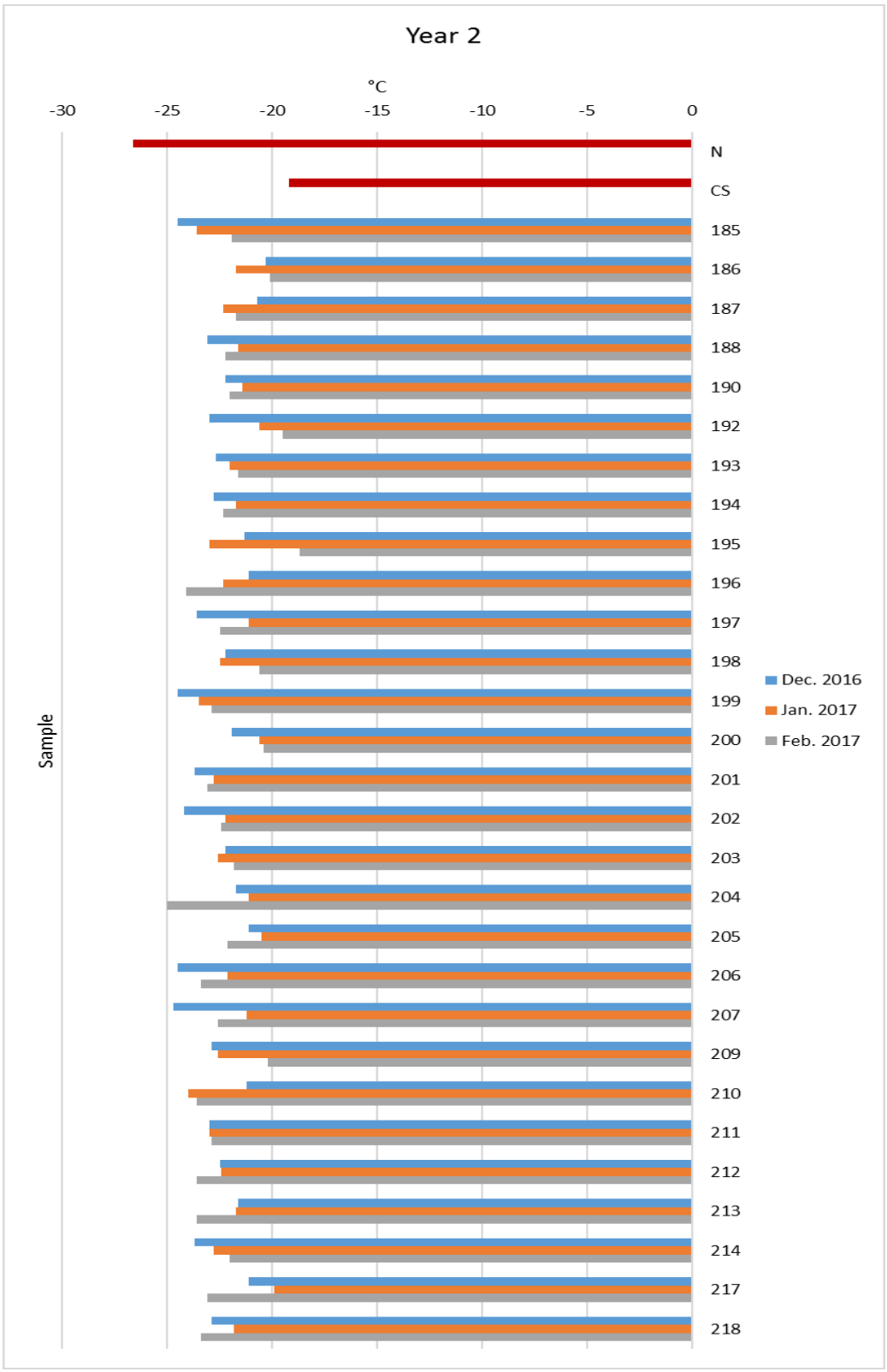

Figure 3 (cont.): Year 2 (2016-2017) Data. This chart shows the average lethal freezing temperature of samples 185-218 within our population for each month of the study alongside the average lethal temperature for the parents.

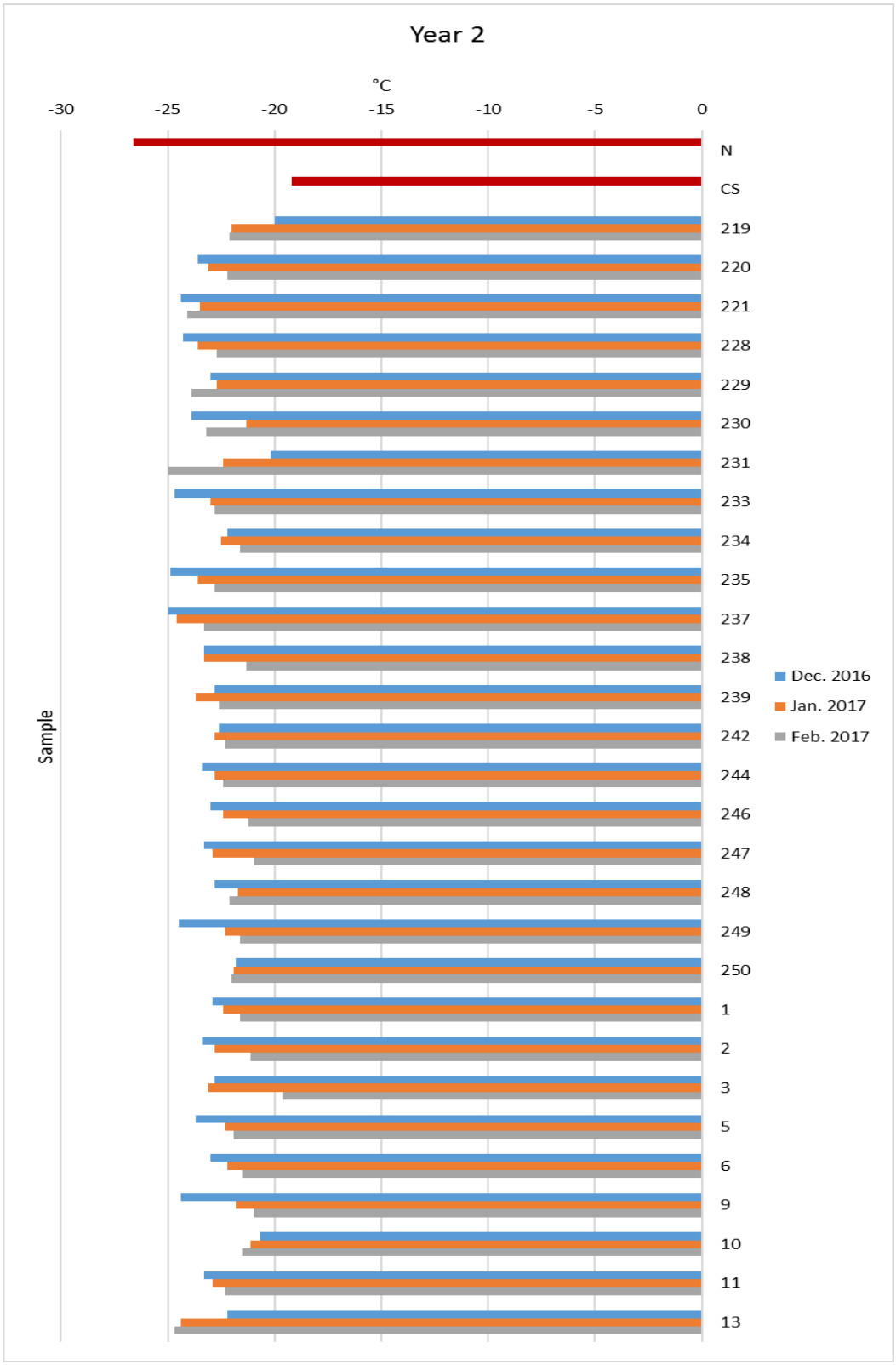

Figure 3 (cont.): Year 2 (2016-2017) Data. This chart shows the average lethal freezing temperature of samples 219-13 within our population for each month of the study alongside the average lethal temperature for the parents. Sample numbers below 100 represent plants from the 2005 population.

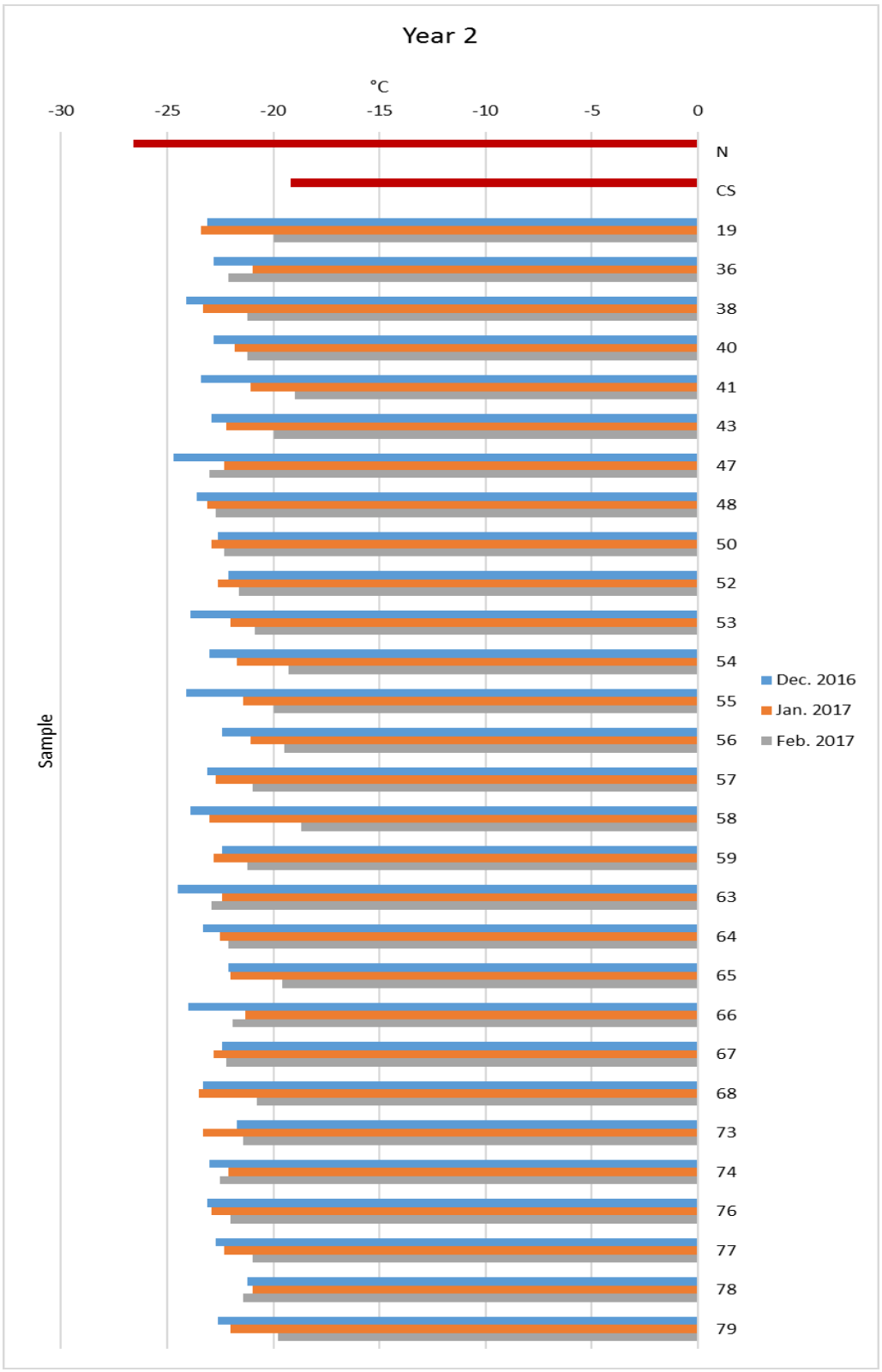

Figure 3 (cont.): Year 2 (2016-2017) Data. This chart shows the average lethal freezing temperature of samples 19-79 within our population for each month of the study alongside the average lethal temperature for the parents.

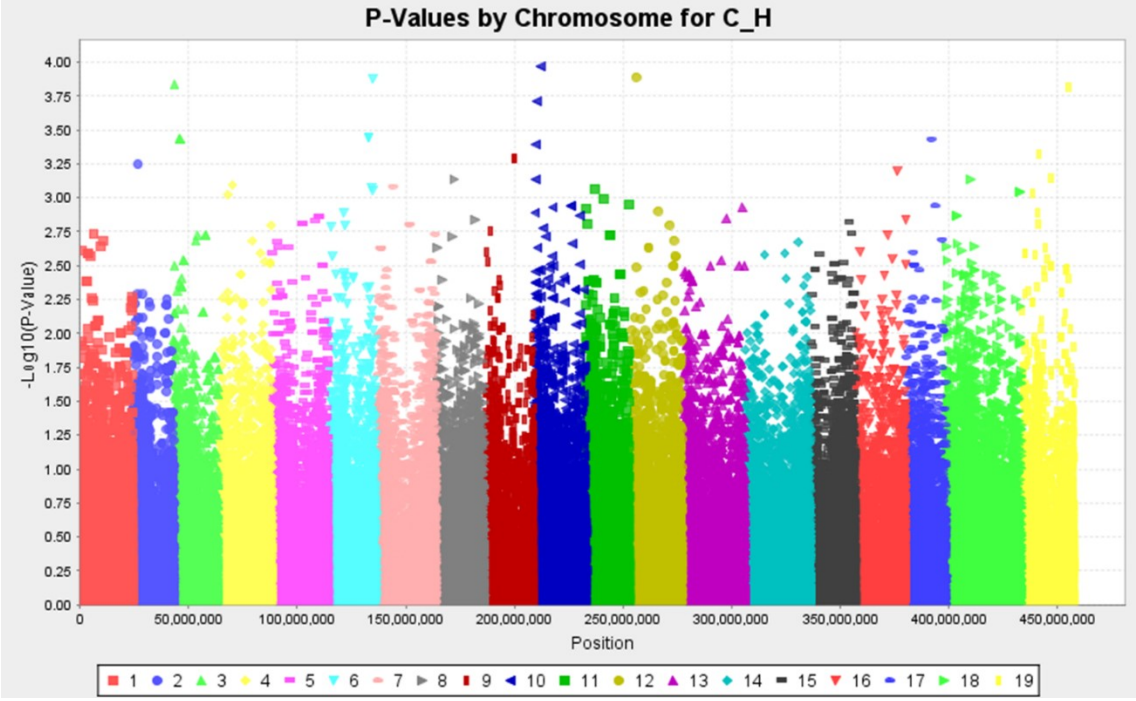

Figure 4: Manhattan Plot of Cold Hardiness Data - Lack of a strong peak shows that no major QTLs are present. Presence of a potential peak at chromosome 10 could indicate one of several minor QTLs. This image was generated using TASSEL to link phenotypic data to the genetic data of our population. Each point represent one SNP within our population, and each bar represents one chromosome.

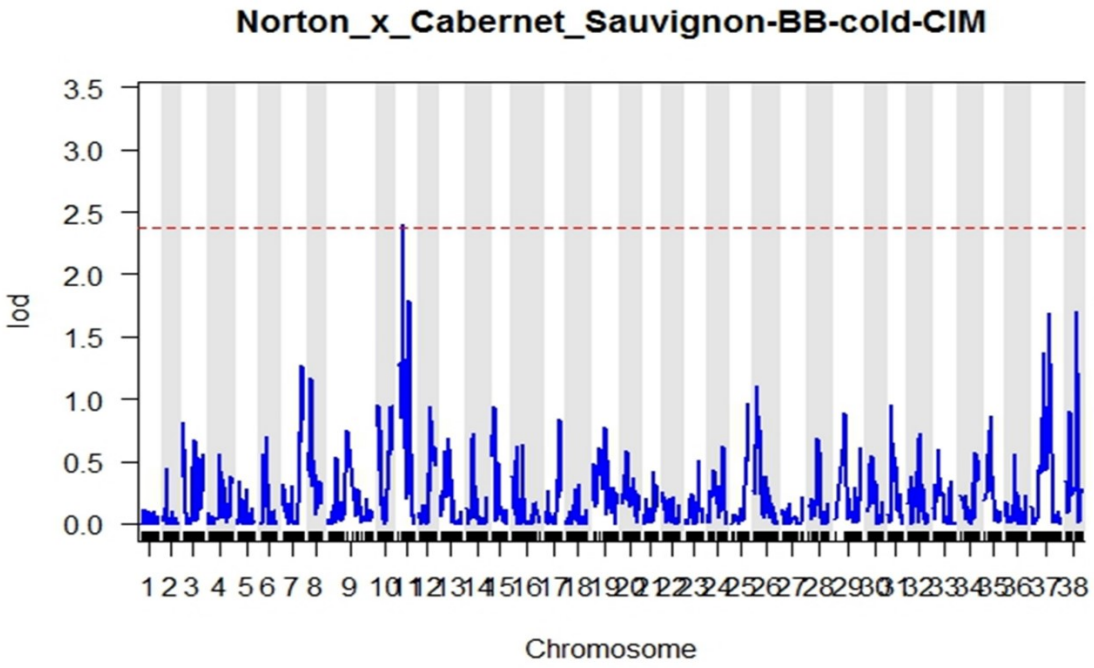

Figure 5: R/QTL Analysis of Cold Hardiness Data - No peak is strong enough to suggest a major QTL; however, the peak at chromosome 10-11 could indicate a minor QTL.

DISCUSSION

Data Limitations

Attempts were made to minimize and remove any sources of error; however, there were some unavoidable risks in this study. One major problem is equipment age and limitations. The equipment used for this project is over twenty years old and as such was more likely to have issues. On several occasions, an entire set of data needed to be collected a second or third time due to equipment malfunctions. These malfunctions include that freezer shutting down before completing the run cycle, the computer not recording data, and faulty wiring causing some sensor to short out. Every effort was taken to prevent these occurrences, the freezer was reprogrammed regularly and broken sensors were avoided, but it is possible that some malfunctions went unnoticed. Additionally, the design of the sample box was such that improperly covered sensors could touch, again causing a short; sensors were carefully wrapped and arranged in the box to avoid this result. The equipment was also limited in the amount of samples it could run at once. The sample box contained thirty sensors and two temperature modules, but one temperature module and one sensor broke after the first few runs. As such, these were avoided in future runs. Unfortunately, because the sample box could only hold twenty-nine sample and a single run took fourteen hours, data collection took roughly one week per month, and the weather could vary greatly over that week. Collecting some samples when the temperature was in 25-35 °F and others when the weather was 50-55 °F can influence the cold hardiness of that genotype and may have skewed the results. Attempts were made to reduce the effect of these varying temperatures by collecting only enough buds for one

run and storing them at 4 °C for 12 hours before data collection. While one set of buds were in the freezer, another set would be taken and stored at 4 °C. In this way, every bud was at the same starting point when the cold hardiness test began, despite the different collection temperatures. Preliminary tests showed that storing the buds in this way reduced the effects of varying temperatures without having a significant impact on the overall cold hardiness of the buds.

Potential errors also arose during the data analysis phase of the project. In some cases, bud peaks on a line were spread across a wide range, or one peak may be far from the others (see Figure 1). These results were not unexpected, as material limitations meant that some buds were much bigger or smaller than other which could produce such a result. This did mean that during the data analysis decisions needed to be made concerning which peaks were relevant to the study. To aid in this decision, relative bud sizes were recorded during the sample preparation phase, and this was compared to the cold hardiness line graph. These outlying peaks occurred most often on plants in which one bud was significantly larger or smaller than the others. When this scenario arose, the outliers were typically removed. The average LT_{50} was then gathered from the remaining peaks. Average values were chosen because they were easier to work with, and the goal was to get an idea of the total cold hardiness for any given genotype in our population. This data was easily analyzed in TASSEL for QTL analysis; however, average hardiness data may not be useful for other, more specific cold hardiness related studies.

Applications

There are many potential applications of a study such as this. In general, cold hardiness studies are of great value to farmers as winter temperatures are a limiting factor for plant growth. Even just measuring the cold hardiness of the parent chosen for this population can reveal important information. Both Norton and Cabernet Sauvignon grapes have been studied before, but few of those studies have taken place in Missouri. With Missouri's growing number of wineries and vineyards, having research data on popular grape cultivars will benefit anyone looking to start their own business. Data such as this can help farmers and future researchers get an idea of what they should plant/study and what kind of performance they might expect from those plants. In terms of plant breeding, the lethal temperatures are now known for the breeding population at Missouri State University's Fruit Research Station. This data can be used on its own to decide which hybrids may be useful for future cold hardiness breeding, or cold hardiness can be combined with the data for disease resistance and rooting ability to find out which hybrids should have the best all-around performance. The better plants can either be studied independently as a potential cultivar or used in other breeding projects.

From a genetic standpoint, this project furthers not only Missouri's current grape breeding program but can be of assistance to anyone working with Norton and its hybrids. There has been a lot of genetic work done in grapes, especially in the more popular and economically important varieties, but relatively little has been done with Norton grapes. Using genetic technologies, specific genes can be linked to traits in Norton hybrids. The associated markers can be used to screen for those genes and, consequently, the desired trait when the plants are still very young (only around 100mg

of plant material is needed for enough DNA to perform PCR then this analysis). This allows breeders to find which hybrids will likely perform in the desired way well before spending the effort to plant and maintain them.

Summary

In conclusion, cold hardiness is one of the most important traits for farmers because it will ultimately determine where a crop can be grown and how well that crop will perform. Because of its importance, cold hardiness has been extensively studied in a wide variety of crops, and many interesting discoveries have been made. Studies have shown that a plant's cold hardiness is at least partially dependent on its area of origin. European grapes are well adapted to a mild climate that is neither too hot nor too cold. American grapes, meanwhile, are better adapted to the climate of states like Missouri and Virginia, where weather varies from season to season and can fluctuate sharply within a season. The differences between European and American grapes makes them ideal parents for new hybrid varieties than will grow well in any part of the United States and still produce a high-quality wine valued by consumers. The goal of this project was to study the cold hardiness of a population of such hybrids and determine what part the genome may be responsible for this trait. This information will prove useful to both farmers and researchers as it can lead to the development of a new cultivar that is ideally suited to Missouri's growing climate. Furthermore, learning which genes are associated with cold hardiness in Norton and its hybrids creates the opportunity to study those genes and their purpose. Such studies can help scientists to better understand how cold

acclimation works and what processes are involved. This in turn can be used to develop even hardier plants or possible methods to improve hardiness in existing varieties.

This study took place over two winter seasons at the Missouri State University Fruit Research Station at Mountain Grove, Missouri, using a breeding population that was established in 2005 and expanded in 2011. Data was collected over the course of one week each December, January, and February. Data was recorded in a spreadsheet and later made into line graphs in which each line represented one plant, and each peak on a line represented one bud from that plant. The average lethal freezing temperature was found for each plant and linked with genetic data that was developed in collaboration with Cornell University through the VitisGene program. This combined data was analyzed with TASSEL to generate a Manhattan plot and locate any major QTLs responsible for cold hardiness. No such QTL was found, suggesting that no single area is primarily responsible for the cold hardiness of our hybrid population. This is not unexpected, as there are many biological, cultural, and environmental factors that alter a plant's overall cold hardiness. Studies have shown that a variety of proteins and chemicals assist grapes during cold acclimation, and it is unlikely that these would all be produced by the same gene. The production of these chemicals and proteins is likely triggered by changing environmental conditions such as day length and ambient temperatures. The data does, however, suggest that there may be minor QTLs associated with cold hardiness, specifically located on Norton's chromosome 10. This particular bar on the Manhattan plot is taller and more consistent that the others but is still not high enough to be considered a major QTL. This is expected because of the nature of cold acclimation. As seen, many components are needed for plants to achieve and maintain

peak cold hardiness. It makes sense then that the genes coding for these components would not all be located in the same position. Additionally, no major QTLs for cold hardiness have been found in grapes at this time, though they have been found for some annual crops. Regardless, the data from this study will be helpful in future Norton grape studies and can be used to aid breeders in choosing better plants at an earlier stage, speeding up a process that would normally take many years.

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